

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**

# Improved lipid-mediated gene transfer in C6 glioma cells and primary glial cells using FuGene™

Bettina Wiesenhofer, Walter A. Kaufmann, Christian Humpel \*

*Laboratory of Psychiatry, Department of Psychiatry, University Hospital Innsbruck, Innsbruck, Austria*

Received 12 January 1999; accepted 18 July 1999

## Abstract

Gene therapy is a potent method to counteract neurodegeneration by introducing genetic information encoding neuroprotective factors. In this study cationic lipids were used to transfer DNA into C6 glioma cells and primary glial cells. When comparing the novel compound FuGene with other commercially-available lipids, it was found that FuGene markedly enhanced gene transfer of a beta-galactosidase reporter plasmid into C6 glioma cells. FuGene had several advantages compared to other lipids, such as a very low toxicity and the capability of transfection under serum conditions. When optimizing, a DNA–lipid ratio of 150 ng DNA/1  $\mu$ l FuGene and a concentration of 3  $\mu$ l FuGene/1 ml medium was found to be optimal. The incubation time peaked after 8 h and the expression time reached an optimum between 2 and 6 days. When cells were transfected on 3 consecutive days for 6 h each ('boosting'), the transfection efficiency was markedly enhanced in primary glial cells. When using endotoxin-free DNA the transfection efficiency could be enhanced up to 3 times. The optimal transfection efficiency in C6 glioma cells and in primary glial cells was found to be  $16.3 \pm 0.3\%$  and  $5.1 \pm 0.37\%$  of total cells, respectively. In conclusion this study shows that the novel compound FuGene has a very high potential to transfer DNA into cells of glial origin, and it might be an interesting candidate for ex vivo and in vivo gene therapeutic approaches. © 1999 Elsevier Science B.V. All rights reserved.

**Keywords:** Astrocytes; Beta-galactosidase; DNA; Gene expression; Gene therapy; Liposomes

## 1. Introduction

Neurodegeneration plays a major role in several human diseases, such as Parkinson's disease or Alzheimer's disease. The gene transfer of neuroprotective molecules may offer a potent method to rescue damaged neurons in the brain (Blacse et al., 1993; Suhr and Gage, 1993; Ridet and Privat, 1995; Karpate et al., 1996). Different approaches are used to introduce protective molecules into the brain: infusion or injection of recombinant purified proteins, transplantation of genetically engineered cells or application of genetic information (Leib and Olivo, 1993; Doering, 1994; Fisher and Ray, 1994; Hefti, 1994; Lindvall and Odin, 1994; Snyder, 1994; Hitchcock, 1995; Ridet and Privat, 1995).

Several methods have been described to introduce genes into mammalian cells in vitro and in vivo: calcium phosphate precipitation, microinjection, electroporation, receptor-mediated gene transfer, gene guns, viral vectors, and lipofection (Murray, 1991). Although no technique has been proven to be most suitable for in vivo gene therapy, lipofection appears to be a promising method (Blacse et al., 1993; Suhr and Gage, 1993; Fisher and Ray, 1994; Ledley, 1995; Ridet and Privat, 1995; Cattaneo et al., 1996; Karpate et al., 1996).

Liposome-mediated transfection (lipofection) is a simple and powerful technique to introduce DNA into mammalian cells (Felgner et al., 1987; Hug and Sleight, 1991; Lasic and Papahadjopoulos, 1995). Liposomes are polycationic lipids, which interact spontaneously and rapidly with polyanions such as DNA and RNA, resulting in liposome/polynucleotide complexes (Felgner et al., 1987). The resulting polycationic complexes fuse with the anionic surface of cells, delivering DNA into the cells via endocytosis. The final transport of DNA into the nucleus is not fully understood.

\* Corresponding author. Present address: Clinic of Psychiatry, Anichstr. 35, A-6020 Innsbruck, Austria. Tel.: +43-512-5043712; fax: +43-512-5043713.  
E-mail address: christian.humpel@uibk.ac.at (C. Humpel)

Several liposome reagents are commercially available and can be used for gene transfer. Recently, we have demonstrated that Lipofectamine™ is a powerful compound to transfer genetic information into cells of the central nervous system (Kofler et al., 1998). However, this lipid is toxic at higher concentrations, works only without serum, and does not successfully introduce DNA into primary glial cells as well as *in vivo*. Thus, the aim of the present study was to examine if other commercially-available lipids, and especially the novel compound FuGene™, may enhance gene transfer of the reporter gene beta-galactosidase into C6 glioma cells and cultured primary glial cells.

## 2. Materials and methods

### 2.1. DNA plasmids

For all lipofection experiments the expression-vector pEF-neo carrying the reporter gene  $\beta$ -galactosidase was used. The  $\beta$ -galactosidase gene was subcloned into a unique NotI site under the control of the strong human elongation factor-1 $\alpha$  promoter (Mizushima and Nagata, 1990). Plasmid DNA was purified using the Quia-gen plasmid purification kit.

### 2.2. C6 glioma cells

C6 glioma cells were cultured at 37°C in 5% CO<sub>2</sub> in culture medium (10% fetal calf serum, DMEM, without antibiotics/antimycotics) and trypsinized two times/week.

### 2.3. Primary glia cells

Nunc plates were pre-prepared by incubation of the wells with 0.5 mg/ml poly-DL-ornithine for 1 h at room temperature. Then the solution was aspirated and the wells were incubated with cell incubation medium (5% horse serum, 0.5% FCS, in optimem1) until use. The whole brains of newborn P0 rats were dissected, washed in PBS + 4 mg/ml DNaseI, and trypsinized for 15 min at 37°C (0.25% trypsin in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free buffer). After addition of 5 ml inactivated horse serum + 4 mg/ml DNaseI, the cells were triturated 20–30 times through a small fire-polished glass pipette. Cells were then triturated through different wide pore needles (0.8–0.4 mm diameter) two times each and filtered twice through a 70  $\mu$ m nylon mesh. The cell number was counted and an aliquot of this cell suspension was added (1500 cells/mm<sup>2</sup>) to the pre-prepared wells. Twice a week fresh medium was added to the cells.

### 2.4. Lipofection with FuGene

FuGene™ is a proprietary blend of lipids (non-liposomal formulation) and other compounds in 80% ethanol.

Cells (10 000 cells/16 mm well) were incubated overnight in medium without antibiotics/antimycotics. To prepare the lipid–DNA complex, 3  $\mu$ l FuGene™ (FuGene; Boehringer Mannheim, Austria) was diluted in optimem1 (Gibco/Lifetech). After incubation for 15 min at room temperature, 450 ng DNA was carefully added, mixed, and incubated at least for 15 min at 37°C. The cells were washed two times in prewarmed optimem1, and then the lipid–DNA mix was carefully (drop by drop) added to the cells. The cells were incubated for 8 h at 37°C/5% CO<sub>2</sub> with the lipid–DNA complex, then the solution was aspirated, and full medium was applied. Cells were normally incubated 2 days at 37°C/5% CO<sub>2</sub> in an incubator. Lipofection with the other commercially-available lipids Lipofectamine™ (Gibco/Lifetech), DOSPER™ (Boehringer Mannheim, Germany), Superfect™ (Qiagen) and Escort™ (Sigma, St Louis, MO, USA) was performed according to the manufacturers instructions.

### 2.5. Trypan blue staining

To determine cell viability as a result of lipid toxicity, trypan blue staining was performed. Briefly, cells were washed in PBS, then incubated in 1.5 ml 0.27% trypan blue solution (in PBS) for 5 min at 37°C. Cells were washed 3  $\times$  with PBS, airdried and mounted in Entellan.

### 2.6. X-gal staining

Cells were rinsed in 100 mM PBS, and then incubated exactly for 5 min on ice with 1 ml fixation solution (2% paraformaldehyde and 0.2% glutaraldehyde in PBS). Cells were washed in PBS, and incubated overnight at 37°C with 1 mg/ml 5-bromo-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal) in color solution (5 mM K<sub>4</sub>[Fe(CN)<sub>6</sub>], 5 mM K<sub>4</sub>[Fe(CN)<sub>6</sub>·3H<sub>2</sub>O], 2 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, PBS). X-gal was resuspended in N-N-dimethyl-formamide before dilution with color solution. Cells were washed in distilled water, airdried, and mounted in Entellan.

### 2.7. Immunohistochemistry

Immunohistochemistry using the avidin-biotin staining technique was performed (Humpel et al., 1996). Cells were washed with 100 mM PBS, fixed for 5 min with 4% paraformaldehyde, rinsed 3  $\times$  in PBS for 10 min at room temperature and pretreated 30 min with 5% methanol/0.3% H<sub>2</sub>O<sub>2</sub>/PBS. Then the cells were rinsed again in PBS, blocked with 10% horse serum/

PBS and then incubated with the primary antiserum (GFAP 1:80, Sigma, St Louis, MO, USA) in a humid chamber overnight at room temperature. Sections were again washed and incubated with secondary anti-rabbit biotinylated antibody (1:200, Vectastain) for 1 h at room temperature. After washing, sections were incubated in Vectastain ABC-reagent for 60 min, washed and the signal detected using 0.5 mg/ml 3,3'-diaminobenzidine (DAB) as a substrate. Slides were air-dried and mounted in Entellan. For  $\beta$ -galactosidase immunohistochemistry, cells were postfixed for 5 min with 4% paraformaldehyde/PBS, rinsed in PBS, pretreated as described above, and incubated with a biotinylated anti- $\beta$ -galactosidase antibody (1:1000, Sigma, St Louis, MO, USA) in a humid chamber overnight at room temperature. Then sections were washed again and directly incubated with Vectastain reagent and processed for DAB detection as described above.

### 2.8. Quantitative measurements

Cells were counted under the microscope using a 1 mm<sup>2</sup>-scale. Blue cells were counted in five randomly-selected areas. Values were averaged and the total number of positive cells per area was calculated. Sections were also analyzed using a computer-assisted image analysis system (Image pro Plus Software, connected to an Olympus BX60 microscope via a Sony video camera). Representative sections were quantified on a gray scale between 0 and 255, where 0 represents black and

255 white. To obtain specific values, unspecific background values were subtracted from the detected specific signal. Multistatistical analysis was obtained by one way ANOVA with subsequent Fisher PLSD posthoc test.

## 3. Results

### 3.1. Different lipids

By testing different commercially-available lipids (Lipofectamine, Dospcer, FuGene, Escort, Superfect), it was found that FuGene was by far the most potent compound to transfer DNA into C6 glioma cells (Fig. 1). Under all three conditions (with and without serum, low, and high lipid concentration) FuGene was better as the other lipids (Fig. 1). Lipofectamine and Escort seemed to be toxic at higher lipid concentrations (Fig. 1).

### 3.2. Cell viability

When comparing Lipofectamine and FuGene on their ability to be toxic for C6 cells, it was found that Lipofectamine started to become toxic at a concentration of 10–12  $\mu$ g/ml, however, FuGene did not significantly affect cell viability, even at a concentration of 14  $\mu$ g/ml medium (Fig. 2).

### 3.3. Optimal standard conditions

To optimize FuGene-mediated parameters, different lipid concentrations were tested with a constant DNA concentration (DNA = 0.2  $\mu$ g/ml). The lipid concentration was found to be optimal at concentrations of > 3  $\mu$ l FuGene/ml medium and did not markedly affect gene-transfer efficiency up to 12  $\mu$ l FuGene/ml medium (Fig. 3A). Using the optimal lipid concentration (3.4  $\mu$ l/ml) the DNA/FuGene ratio was tested. The optimal DNA/FuGene ratio was found to be between 100 and 160 ng DNA per 1  $\mu$ l FuGene (Fig. 3B); the efficiency was dramatically decreased at lower ratios (< 50 ng DNA/1  $\mu$ l FuGene) or at higher ratios (> 160 ng DNA/1  $\mu$ l FuGene). Under optimal conditions (3.4  $\mu$ l FuGene + 510 ng DNA per 1 ml) the optimal cell density was tested: at least 20 000 cells/16 mm well are necessary for optimal gene transfer (Fig. 3C) compared to Lipofectamine. When cells were lipofected with different complex intensities (1x = 1  $\mu$ l FuGene + 150 ng DNA), it was found that 3x complexes (3  $\mu$ l FuGene + 450 ng DNA), significantly enhanced gene-transfer efficiency (Fig. 3D). However, when higher complex intensities (> 4x) were used, markedly decreased gene-transfer efficiencies were found, which seemed to be a result of cell detachment and toxicity (Fig. 3D). The

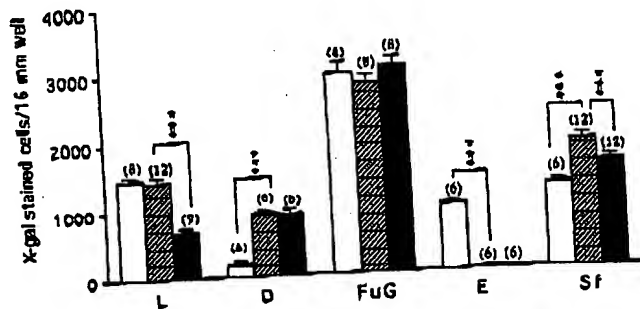


Fig. 1. Different commercially-available lipids (Lipofectamine™ (L), Dospcer™ (D), FuGene™ (FuG), Escort™ (E) and Superfect™ (Sf)) are compared. C6 glioma cells (12 000 cells/16 mm well) were lipofected for 8 h using a constant lipid-DNA ratio (10  $\mu$ g lipid + 600 ng DNA/ml) and  $\beta$ -galactosidase expressing cells were visualized after 48 h using X-gal staining. Lipofection was performed either without serum (open bars) or with 10% serum (hatched bars). Another lipofection experiment was performed with 30  $\mu$ g lipid and 1.7  $\mu$ g DNA under serum conditions (filled bars). Note that FuGene was the most potent lipid under serum and serum-free conditions. Statistical analysis was performed by one way ANOVA with a subsequent Fisher-PLSD posthoc test (\*\*\* $P$  < 0.001). Values are given as number of X-gal stained cells per 16 mm well. Values in parenthesis give the number of experiments.

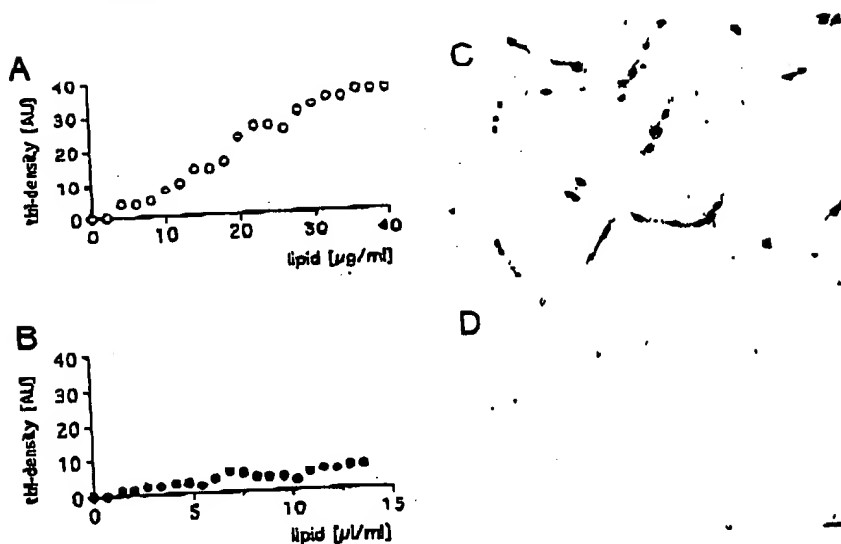


Fig. 2. Cell viability after incubation with Lipofectamine™ (A, C) or FuGene™ (B, D). C6 glioma cells were incubated for 8 h with different concentrations of the respective lipids, and then processed for Trypan blue (tbl) staining. The intensity of staining was analyzed using a computer-assisted image analysis system. Values are given as arbitrary units (AU). Note that the FuGene concentration is given as  $\mu\text{l}$  FuGene/1 ml medium. Fig. C and D show trypan-blue stained cells after incubation with 30  $\mu\text{g}/\text{ml}$  Lipofectamine (C) or 14  $\mu\text{l}/\text{ml}$  FuGene (D). Bar = 130  $\mu\text{m}$ .

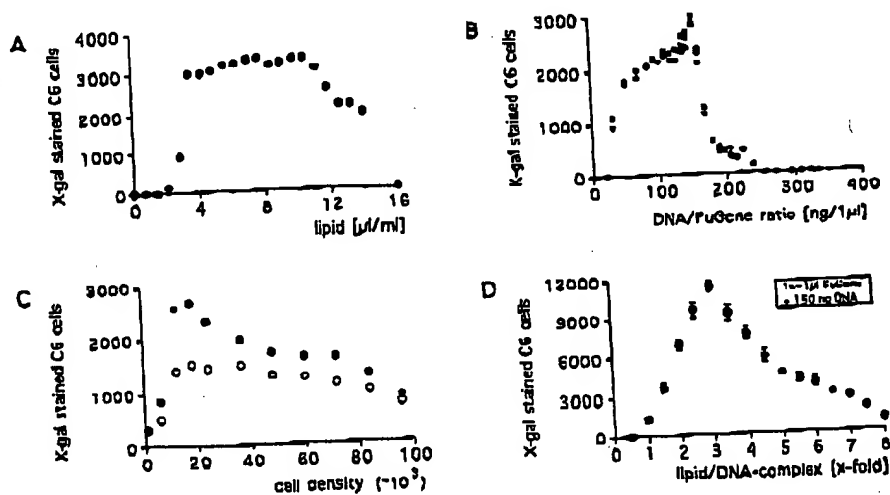


Fig. 3. The lipofection parameters were tested in C6 glioma cells using FuGene. (A) The optimal FuGene concentration was tested using a constant empirically determined DNA concentration (0.2  $\mu\text{g}/\text{ml}$ ). Note that the minimal essential FuGene volume is 3  $\mu\text{l}/\text{ml}$ . (B) The optimal DNA/FuGene ratio was tested using a constant FuGene concentration as found in experiment A (= 3.4  $\mu\text{l}$  FuGene/ml). Note that the lipofection efficiency reaches a maximum between 100 and 160 ng DNA/1  $\mu\text{l}$  FuGene. (C) The optimal cell density was tested under optimized conditions using either Lipofectamine (open circles; 10  $\mu\text{g}/\text{ml}$  Lipofectamine + 600 ng/ml DNA) or FuGene (closed circles; 3.4  $\mu\text{l}/\text{ml}$  FuGene + 510 ng/ml DNA). Note that 20 000 cells/16 mm well are necessary for efficient gene transfer. (D) The optimal FuGene/DNA complex intensity was tested under optimal conditions (1x = 3.4  $\mu\text{l}/\text{ml}$  FuGene + 510 ng/ml DNA; 2x = 6.8  $\mu\text{l}$  FuGene + 1020 ng/ml DNA; 3x = 10.2  $\mu\text{l}/\text{ml}$  FuGene + 1530 ng/ml DNA...). Values are given as the number of X-gal stained cells/16 mm well. Each value represents one independent experiment (A–C). Values are given as mean  $\pm$  S.E.M. (D).

efficiency of gene transfer increased linearly when the C6 cells were incubated with the FuGene/DNA complex and reached a maximum after 8 h, which did not markedly change up to 50 h (Fig. 4A). The expression of  $\beta$ -galactosidase was found to reach a maximum already after 2 days and did not differ up to 7 days;

however, it rapidly decreased after 8 days of incubation (Fig. 4B). When gene-transfer experiments were performed on glass, the lipofection efficiency was dramatically reduced (up to 6-times in C6 cells and up to 36-times in primary glial cells). When testing the stability of the lipid–DNA mixture, it was found that the

complex was stable at 37°C for at least 1 day independent of the preparation in wells or Eppendorf tubes (Table 1). However, the stability of the complex markedly decreased when incubated for 2–3 days at 37°C, which was more pronounced when prepared in

wells (Table 1). Immunohistochemical analysis revealed strong GFAP-staining in C6 glioma as well as primary glial cells (Fig. 5A–C). Under optimal standard lipofection conditions several strong  $\beta$ -galactosidase expressing cells were seen when visualized with X-gal staining (Fig. 5D–F) or  $\beta$ -galactosidase immunohistochemistry (Fig. 5G–J).

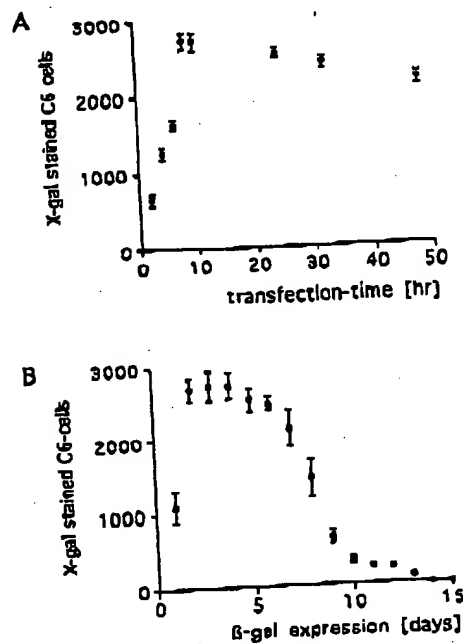


Fig. 4. Gene transfer transfection time (A) and expression time (B) were optimized in C6 glioma cells using FuGene. C6 glioma cells were transfected (3.4  $\mu$ l/ml FuGene + 510 ng/ml DNA) for different transfection times and the efficiency was found to reach a maximum already after 8 h of incubation (A). The  $\beta$ -galactosidase expression increases rapidly, reaches a maximum between 2 and 6 days after transfection and progressively declines after 7–8 days of incubation. Values are given as the mean  $\pm$  SEM X-gal stained cells per 16 mm well ( $N=3-6$ ).

Table 1  
Stability of the lipid–DNA complex<sup>a</sup>

Days	Well	P#	EPP	P*	P§
0	3088 $\pm$ 84	---	2822 $\pm$ 77	---	n.s.
1	2416 $\pm$ 134	***	2715 $\pm$ 96	n.s.	n.s.
2	1153 $\pm$ 93	---	2495 $\pm$ 144	*	***
3	261 $\pm$ 130	---	2076 $\pm$ 131	---	---

<sup>a</sup> FuGene (1.7  $\mu$ l) was mixed with DNA (255 ng) in 500  $\mu$ l optimehl and incubated for 0, 1, 2, or 3 days at 37°C, then lipofected into C6 cells and analyzed after 48 h. The lipid–DNA complex was either prepared in polystyrene Nunc 24 well plates (WELL) or in polypropylene Eppendorf tubes (EPP). Values are given as X-gal stained cells/16 mm well. The number of experiments was 7–12. Statistical comparison was performed by one way ANOVA with subsequent Fisher PLSD test. P# and P\* give the differences between the well and Eppendorf groups, respectively, compared against the 0 day group. P§ compares the well and Eppendorf groups of the same days (\* $P<0.05$ ; \*\* $P<0.01$ ; \*\*\* $P<0.001$ ; n.s. = not significant).

### 3.4. Endotoxin-free DNA and boosting

When C6 cells were transfected with endotoxin-free DNA under serum-free conditions, the transfection efficiency was significantly enhanced (Fig. 6A). However, the effects of endotoxin-free DNA were abolished under serum-containing conditions (Fig. 6A). When primary glial cells were transfected on 3 consecutive days for 6 h each ('boosting') under serum-free conditions, the transfection efficiency was significantly enhanced when compared to standard (1  $\times$  8 h) conditions (Fig. 6B). When endotoxin-free DNA was used the transfection efficiency was significantly increased compared to normal (not endotoxin-free) DNA (Fig. 6B).

### 3.5. Gene-transfer efficiencies

All experiments with C6 cells were started with  $10\,600 \pm 1200$  cells/16 mm well ( $N=10$ ), a number which increased to  $20\,500 \pm 1800$  cells/16 mm well ( $N=10$ ) overnight. After 8 h of lipofection and an additional incubation time of 48 h, the total number of cells/16 mm well was calculated as  $57\,000 \pm 6400$  cells/16 mm well ( $N=9$ ). According to this number of C6 glioma cells, the gene-transfer efficiency under standard conditions (8 h lipofection and optimal DNA/FuGene ratio) was found to be  $5.7 \pm 0.2\%$ , and significantly increased up to  $16.3 \pm 0.6\%$  using endotoxin-free DNA. The efficiency for gene transfer in primary glial cells was  $5.1 \pm 0.3\%$  under optimal 'boosting' conditions.

## 4. Discussion

This study shows that the novel transfection reagent FuGene markedly enhanced gene transfer into C6 glioma cells and primary glial cells. It was found to be important to optimize lipid and DNA concentrations as well as the expression and incubation times. The use of highly-purified endotoxin-free DNA significantly increased the gene-transfer efficiency.

Cationic liposome-mediated DNA transfer appears to be a promising method for CNS gene transfer (Loeffler and Behr, 1993; Fisher and Ray, 1994; Ridet and Privat, 1995). Recently, we demonstrated (Kofler et al., 1998) that Lipofectamine™ was potent to transfer DNA into C6 glioma cells and primary glial cells, in vitro but not in vivo. The present study extends our

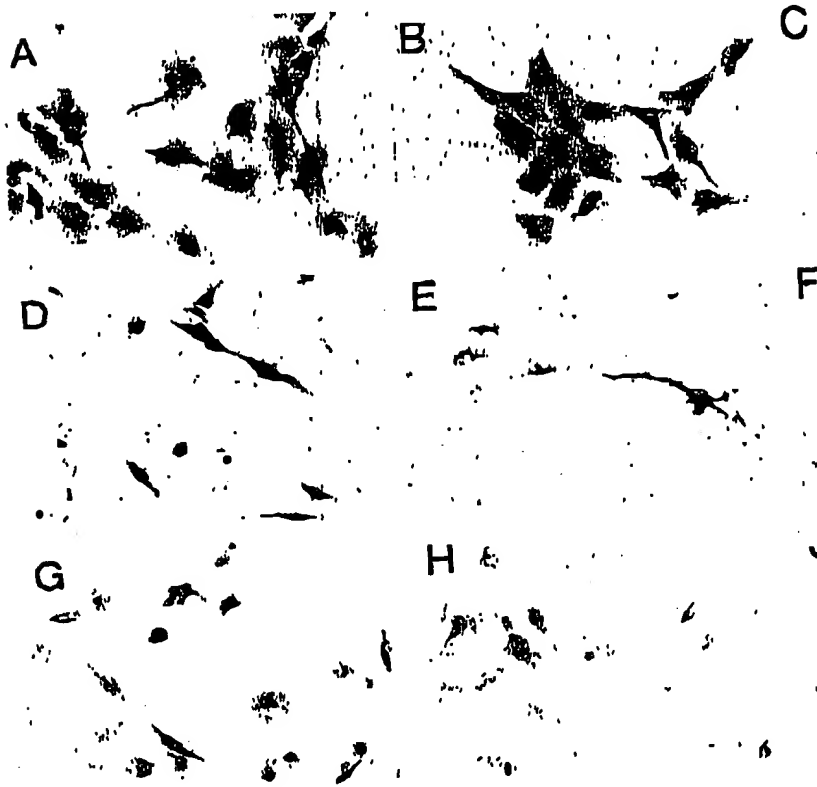


Fig. 5. C6 glioma cells (A) and primary glial cells (B) were immunohistochemically stained using a GFAP antibody. Negative controls are C6 glioma cells where the primary antibody was omitted (C). C6 glioma cells (D) and primary glial cells (E) were transfected under optimal conditions (3.4  $\mu$ l/ml FuGene + 510 ng/ml DNA, 8 h) and visualised 2 days after transfection. Negative controls are C6 glioma cells which were incubated only with FuGene (F). C6 glioma cells (G) and primary glial cells (H) were transfected under optimal conditions and the  $\beta$ -galactosidase expressing cells stained with a biotinylated anti- $\beta$ -GAL antibody. Negative controls are glioma cells, where the antibody was omitted (I). Bar = 35  $\mu$ m.

previous findings and shows that other lipids are equally effective to Lipofectamine™, but the novel lipid FuGene exhibited a marked advantage over the other compounds. Successful transfection is dependent upon multiple experimental parameters, such as the quality of DNA, nature of cell lines, contaminations, growth requirements, and other parameters. While the reproducibility with Lipofectamine™ was low, the reproducibility using FuGene was excellent. However, optimization of FuGene-mediated transfection parameters is necessary and critical, because the lipid–DNA ratio has a narrow window for optimal gene transfer. Our data show that too low as well as too high DNA concentrations markedly decreased gene-transfer efficiency. This might be explained because an incorrect concentration of polyanionic DNA may not form correct DNA–lipid complexes, so that, e.g. the outer surface of the complex does not become polycationic and may lose the binding capability to the anionic surface of cell membranes. Also the property of FuGene to deliver DNA in the presence of serum into the cells is a marked advantage over other lipids. This might allow to continuously

incubate the cells with the transfection solution for a long time.

When testing the stability of the lipid–DNA complex, we observed no loss of activity for up to one day kept at 37°C. However, the activity decreased when the mix was kept for more than 1–2 days at 37°C. This decrease was more pronounced when the mix was prepared in wells compared to Eppendorf tubes resulting in marked loss of gene-transfer activity when kept for 3 days in wells. This seems to be because of the fact that wells are not closed and that the diffusion rate is markedly higher than in closed Eppendorf tubes. Such a diffusion may change the lipid–DNA composition or alternatively oxidative damage of lipid–DNA may occur.

While several of the lipids, including Lipofectamine, are highly toxic at higher concentrations (Felgner et al., 1987; Kofler et al., 1998), FuGene did not exhibit any toxicity even at very high concentrations. That makes this lipid an important tool in gene delivery in vivo. Although FuGene alone was not toxic up to 14  $\mu$ l, the lipid–DNA complex revealed an increased toxicity.

This was interesting to note, because such findings may have important implications for in vivo gene-transfer experiments. Our data show, that the Fugene-DNA complexes linearly increased the gene-transfer efficiency; however, more than 3-times of the complex intensity resulted in a marked detachment of the cells from the petri dishes. Complex intensities of about 7-8 times induced fast cell death. This indicates that although FuGene alone is not toxic, the lipid-DNA complex has other characteristics to affect cells.

When comparing the preparation of the lipid-DNA mix in polystyrene (wells) and polypropylene (Eppendorf) tubes, no differences were found on gene-transfer efficiency. However, transfection in glass chamber slides revealed markedly reduced transfection efficiencies. In fact Felgner (Felgner et al., 1987) reported that lipid-DNA complexes are very sticky and can adhere to glassware but not to polystyrene. Thus, our data are in full line with these reports and highly suggest the use of

polystyrene petri dishes and plates for liposome-mediated gene transfer. This is in line with our previous study (Kofler et al., 1998), where Lipofectamine™ was used to transfect primary glial cells growing in glass chamber slides, which revealed only a very low transfection efficiency. Thus, this observation is very important for performing in vivo gene-transfer experiments using glass microsyringes.

Lipofection into primary cells has been shown to be very complicated and yielded very low transfection efficiencies with Lipofectamine™ (Kofler et al., 1998). Yang et al. (1994) reported that approx. 1000 cells per 16 mm well of a septo-hippocampal cell culture were transfected, a number we could never reach in our previous study when using Lipofectamine (Kofler et al., 1998). Kaech et al. (1996) reported transfection efficiencies of about 1-3% for primary hippocampal neurons in culture being more close to our previous results. However, our present study shows that transfection of primary glial cells using FuGene was markedly enhanced and reached transfection efficiencies of up to 5.1% (approx. 3000 cells/16 mm well).

The protein expression after transfection with a circular plasmid was maximal between 2 and 6 days after lipofection but decreased rapidly thereafter, indicating transient transfection. Such a time course is in line with others (Yang et al., 1994) and has also been shown by us recently using Lipofectamine™ (Kofler et al., 1998). Such a transient time course might be a major problem for long-term gene therapy studies, where continuous effects of a specific protein of interest is expected. However, on the other hand, transient systems allow better control over the transfected gene, which result in unexpected side effects. The use of on-off gene expression vectors, or episomal vectors, as well as linear-integrating vectors may help to overcome the problems of transient expression.

Endotoxins (also known as lipopolysaccharides) are cell membrane components of gram-negative bacteria such as *Escherichia coli* and are released during the lysis step of plasmid purification. Endotoxins can be toxic to primary cells and may affect gene transfer (Cotten et al., 1994). In fact, it has been shown that up to 500 endotoxin units/ $\mu$ g DNA decreased the transfection efficiency in 3T3 cells to 50% (White et al., 1998). Our data show, that the use of pure endotoxin-free DNA is important in gene transfer, especially when using primary glial cells but not C6 glioma cells. An explanation may be that postnatal cells are very sensitive against different toxic environments, while malignant tumor cell lines are very resistant. In addition, we show that serum can inhibit the effects found with endotoxin-free DNA, which may indicate that serum itself contains endotoxins or other factors, which negatively affect the gene transfer.

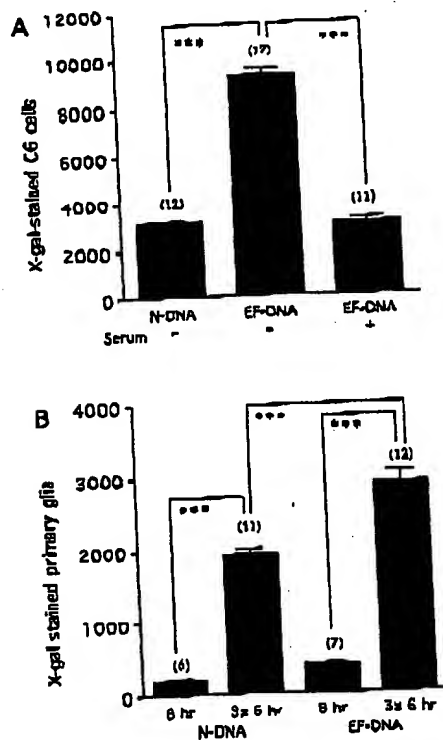


Fig. 6. Increased gene-transfer efficiencies using endotoxin-free DNA and 'boosting' conditions. C6 glioma cells (A) were transfected using normal (not endotoxin-free DNA; N-DNA) or using endotoxin-free DNA (EF-DNA) under serum-free (-) or serum-containing (+) conditions. Primary glial cells (B) were transfected under serum-free conditions using either standard conditions (1 x 8 h) or boosting-conditions (3 x 6 h) with EF-DNA and N-DNA. Values are given as mean  $\pm$  SEM X-gal stained cells/16 mm well. The number of experiments are given in parenthesis. Statistical analysis was performed using one way ANOVA with subsequent Fisher PLSD test (\*\*\* $p$  < 0.001).



'Boosting' is a technique where the transfection is amplified by application of the lipid-DNA mix on 3 consecutive days. Transfection was performed for 6 h under serum-free conditions, and followed immediately by an overnight incubation in serum medium, which reduces cell death as a result of serum withdrawal. In fact, such a 'boosting' markedly enhanced the gene-transfer efficiency in primary cells. It is suggested that a repeated lipofection enhances the chance to transfer genes into cells, which were not transfected during the first or second application.

In summary, we show that the novel compound FuGene markedly enhanced the gene transfer when compared to other commercially-available lipids. Gene-transfer efficiencies were highly dependent on the correct lipid-DNA ratio and on using endotoxin-free DNA. In conclusion, FuGene is a novel compound which has several advantages over other commercial lipids and may be an important tool to transfer DNA into glia or neurons in vivo to possibly counteract neurodegenerative insults.

#### Acknowledgements

This study was supported by the Austrian Science Foundation (P11956MED).

#### References

- Blaese RM, Mullen CA, Ramsey WJ. Strategies for gene therapy. *Pathol Biol* 1993;41:672–6.
- Cartoneo E, Conti L, Gritti A, Frollichsthal P, Govoni S, Vestovi A. Nonvirally mediated gene transfer into human central nervous system precursor cells. *Mol Brain Res* 1996;42:161–6.
- Cotten M, Baker A, Saltik M, Wagner E, Buschle M. Lipopolysaccharide is a frequent contaminant of plasmid DNA preparations and can be toxic to primary human cells in the presence of adenovirus. *Gene Therap* 1994;1:239.
- Doering LC. Nervous system modification by transplants and gene transfer. *BioEssays* 1994;16:825–31.
- Foligner PI, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, et al. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci USA* 1987;84:7413–7.
- Fisher LJ, Ray J. In vivo and ex vivo gene transfer to the brain. *Curr Opin Neurobiol* 1994;4:735–41.
- Hefli F. Neurotrophic factor therapy for nervous system degenerative diseases. *J Neurobiol* 1994;25:1418–35.
- Hitchcock E. Current trends in neural transplantation. *Neurol Res* 1995;17:33–7.
- Hug P, Sleight RG. Liposomes for the transformation of eukaryotic cells. *Biochim Biophys Acta* 1991;1097:1–17.
- Humpel C, Marksteiner J, Saria A. Glial-cell-line-derived neurotrophic factor enhances biosynthesis of substance P in striatal neurons in vitro. *Cell Tissue Res* 1996;286:249–55.
- Kaech S, Kim JB, Carlola M, Rulston E. Improved lipid-mediated gene transfer into primary cultures of hippocampal neurons. *Mol Brain Res* 1996;35:344–8.
- Karpati G, Lochmuller H, Nalbantoglu J, Durham H. The principles of gene therapy for the nervous system. *TINS* 1996;19:49–54.
- Kofner P, Wiesenhofer B, Rehr C, Baier G, Stockhammer G, Humpel C. Liposome-mediated gene transfer into established CNS cell lines, primary glial cells, and in vivo. *Cell Transplant* 1998;7:175–85.
- Lasio DD, Papahadjopoulos D. Liposomes revisited. *Science* 1993;267:1275–6.
- Ledley FD. Nonviral gene therapy: the promise of genes as pharmaceutical products. *Human Gene Ther* 1995;6:1129–44.
- Leib DA, Olivo PD. Gene delivery to neurons: is herpes simplex virus the right tool for the job? *BioEssays* 1993;15:547–54.
- Lindvall O, Odin P. Clinical application of cell transplantation and neurotrophic factors in CNS disorders. *Curr Opin Neurobiol* 1994;4:752–7.
- Loeffler JP, Behr JP. Gene transfer into primary and established mammalian cell lines with lipopolyamine-coated DNA. *Methods Enzymol* 1993;217:599–619.
- Mizushima S, Nagata S. pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res* 1990;18.
- Murray EJ. *Gene Transfer and Expression Protocols*. Clifton: Humana Press, 1991.
- Ridet JL, Privat A. Gene therapy in the central nervous system: direct versus indirect gene delivery. *J Neurosci Res* 1995;42:287–93.
- Snyder KY. Grafting immortalized neurons to the CNS. *Curr Opin Neurobiol* 1994;4:742–51.
- Suhr ST, Gage FH. Gene therapy for neurologic disease. *Arch Neurol* 1993;50:1252–68.
- White D, Butler B, Crosswell D, Smith C. Wizard PureFecton plasmid DNA purification system: the new standard in isolating transfection grade plasmid DNA. *Promega Notes* 1998;68:2–6.
- Yang K, Faustiniella F, Xue JJ, Whitson J, Kampfl A, Mu XS, et al. Optimizing liposome-mediated gene transfer in primary rat septo-hippocampal cell cultures. *Neurosci Lett* 1994;182:287–90.